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LASER FLOW CYTOPHOTOMETRIC LIGHT SCATTER AND FLUORESCENCE TIME-OF-FLIGHT SIZING OF MAMMALIAN CELLS

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ABSTRACT

In laser flow cytophotometry, an increasingly popular technique of analytical cytology, quantitative measurements of interest include cell and nuclear diameters. Electronic circuitry for a new method of time-of-flight cell sizing has been developed which measures the time that signal pulses from either fluorescence or light scatter sensors exceed a preset constant fraction of the peak signal amplitude (pulse width) or the time that it takes a signal to rise between constant fractions of the peak signal amplitude on the rising side of the pulse (pulse rise-time). These pulse width or pulse rise-time measurements were related to cell or nuclear diameters and were used in combination to determine nuclear size to cell size ratios. This new type of time-of-flight sizing was found to be independent of fluorescent or light-absorbing stain intensity, linearly related to cell or nuclear diameter, and capable of resolving diameter differences of less than 0.5 μm.

INTRODUCTION

Previous attempts to determine cell or nuclear size with flow cytometers have measured one or more of the following parameters: intensity of light scattered at small angles (8), absorption of incident light (2), or time-of-flight (TOF) sizing using a laser beam width larger than the diameter of the cell being measured (9). The intensity of the light scattered in the near forward direction is predominately due to diffraction and is roughly proportional to the cross sectional area of the cell. The angular width of this first ring of the diffraction pattern, however, decreases with increasing cell size (3). The use of laser beam dumps or obscuration bars in commercial flow cytophotometers modifies the proportion of this scattered light that reaches the detectors thereby leading to a nonsystematic relationship between scattered light intensity and cell cross sectional area (3). At angles greater than those subtended by the main diffraction ring of the cell, typically 2^{o} - 3^{o} for mammalian cells, the scattered light intensity is strongly dependent on cell refractive index. Two cells of the same size but of different refractive index will have different light scatter intensities, so that light scatter intensity is not a reliable measure of cell size.

To date, time-of-flight sizing techniques in flow cytophotometers have employed a fixed voltage threshold triggering technique that measures the length of time a cell's light scatter or fluorescence signal is above a fixed, predetermined voltage threshold (9, 12). Sharpless (6,7) has shown that measurements of cell size based on the time a signal is greater than a fixed voltage threshold has a strong, nonlinear dependence on the amplitude of the signal as well as its duration. Since a low amplitude (e.g. from a dimly fluorescent cell), signal is above a fixed threshold for a shorter time

than a higher amplitude signal, cells of the same size will produce different pulse widths. This situation is clearly unsatisfactory for biological experiments in which the staining intensity responsible for either the light scatter or fluorescent signal may vary considerably among cells of the same size.

The objective of this work is to accurately size cells and nuclei independently of the intensity of fluorescent or light-absorbing stains used to measure other cellular properties. In order to accomplish this objective a new method of time-of-flight sizing which allows high resolution, amplitude independent sizing of cells or nuclei with laser beam widths larger than the cell diameter was developed. This permits simultaneous light scattering analysis of cell structure and high resolution sizing of small biological objects. This was accomplished by developing pulse analysis circuitry to measure pulse widths at fixed fractions of pulse height, as suggested by Sharpless (7), and pulse rise times.

MATERIALS AND METHODS

Laser flow Cytometry

Commercial flow cytometers used with this time-of-flight system were the Ortho Instruments (Westwood, Mass.) Cytograf (Model 6300) and Cytofluorograf (Model 4802A) and the Becton-Dickinson (Mountain View, Calif.) FACS II.

Amplitude Independent Cell Sizing

Electronic circuitry was designed and constructed for the pulse height independent measurement of pulse width or pulse rise time. In measuring the transit time of a cell through a laser beam (time-of-flight sizing) (Fig. 1), the peak of the incoming voltage pulse from the detectors is sensed and held for 70 μsec. During this time voltage dividers take percentages of the peak amplitude according to preset threshold levels and mode of operation (pulse width or pulse rise-time) desired. A high fidelity delay line of 8 μsec (Allen Avionics Electronics, Inc., Mineola, NY) is used to produce a delayed replica of the incoming signal which is subsequently presented to voltage comparators which produce start and stop signals for a time-to-time amplitude convertor (TAC), which linearly charges a capacitor during the time between the start and stop signals. The range of the time-to-amplitude conversion can be adjusted by varying the capacitor charging rate. The threshold and capacitor charging rates are factors which must be adjusted to insure that the output signals are within the voltage level and response time limitations of the electronics used for signal analysis (multichannel analyser or computer). A diagram showing in more detail how the time-of-flight measurements are generated is given in Figure 2.

Simultaneous Intensity and Time-of-Flight Measurements

In certain experiments it was necessary to examine two pulse parameters simultaneously for each cell to provide correlated signal measurements. To accomplish this the output signal from the TOF circuit was digitized and

transmitted to an IMSAI 8080 microprocessor and correlated with the corresponding amplitude TOF signal from either the scatter or fluorescence sensors of the flow cytophotometer. A 3-dimensional display of the cells having two correlated parameters (e.g. fluorescence TOF and light scatter TOF) can be recorded on the teletype by using a 64 x 64 character printout in two dimensions and by presenting the third dimension as the number of cells having a particular pair of values in terms of character symbols from 0 through 9, and A through Z and finally special teletype characters in multiples of N cells. For example, if the multiple s 32 then a printed "2" means 64 cells, and a printed "3" means ls. This display therefore constitutes a digital (3-dimensional) contour map of the two-parameter distribution.

Cells and Viruses for Absorption and Fluorescent Staining

HEp-2, a cell line derived from a human laryngeal carcinoma, was obtained from the American Type Culture Collection and was grown in 16 oz. prescription bottles in Dulbecco's modified minimum essential medium (1), supplemented with 10% heat-inactivated donor calf serum (Grand Island Biological Co., Grand Island, NY), 0.085% $NaHCO_3$, 100 units/ml penicillin, 50 µg/ml streptomycin. Cells were infected with herpes simplex virus type 2, strain 316-D, provided by Dr. John Docherty, The Pennsylvania State University. Virus was allowed to adsorb to the cells for one hour. Unadsorbed virus was then decanted and the cells were given fresh medium and incubated for 18 hours before being fixed in 5% neutral buffered formalin (4). Human embryonic lung cells (HEL) were grown and infected in a similar manner. Viral antigens in the cytoplasm were stained with diaminobenzidine by the Sternberger P-A-P (peroxidase-anti-peroxidase) method (10). Nuclei were stained with acriflavin, a fluorescent stain which is specific for A-T nucleotide pairs (8). A procedure combining these two methods was used to obtain doubly stained cells (3). Acriflavine-stained near-diploid cultured Chinese hamster cells, line M3-1F3, were also

employed. Sizing calibrations were performed using uniformly-sized microspheres (Duke Scientific Co., Palo Alto, California and Coulter Electronics, Hialeah, Florida).

RESULTS

In order to test the amplitude independence of the time-of-flight measurements cultured Chinese hamster cells were stained by differing acriflavin-feulgen staining protocols to yield cells with nuclei of similar sizes but of different fluorescent intensities. Figure 3 shows that the time-of-flight size of the nuclei of cells in the G_2 phase of the life cycle is independent of fluorescent signal amplitude over the range of fluorescent intensities provided by this type of experiment.

Time-of-flight measurement increases linearly with particle diameter. The data of Fig. 4 indicate achievable resolution of 1.0 μm per channel using pulse width measurements, and the data of Fig. 5 indicate achievable resolution of 0.2 μm per channel using pulse rise time measurements on calibrated microspheres 5 - 20 μm in diameter.

Nuclear diameter distributions can be measured by pulse rise TOF, and they reflect the life cycle distributions of exponentially growing human cells (Fig. 6a) and the distorted nuclear size distributions of virus-infected cells (Fig. 6b).

Nuclear diameter to cell diameter ratio, a parameter commonly used in analytical cytology, can be measured with paired TOF circuits and a microprocessor or two parameter analyser. Figure 7 diagrams the method of this measurement on cells with stained and unstained cytoplasm. To demonstrate this principle the nuclei of fixed virus-infected and uninfected HEp-2 cells were stained with acriflavine, and their cytoplasms were stained for virus antigens. The correlated 2-parameter distributions are shown as teletype output in Fig. 8. The distributions in the left panel were obtained with HSV-infected cells and show two populations, A and B, with N/C ratios distorted relative to those of normal cell populations, D and E, in the right panel. The control cell preparation also contained bare nuclei, which had

the expected N/C ratio of nearly 1.0. The absolute values of the N/C ratios were calculated using calibration curves similar to those shown in Figs. 4 and 5.

DISCUSSION

We have described the construction and testing of a pulse height independent TOF cell sizing device for laser flow cytophotometers This device implements the notion introduced by Sharpless (7) that pulse width measured at a fixed fraction of pulse height (rather than at a fixed voltage level) would be insensitive to the absolute intensity of light scattering or fluorescence of the cell. We have added the notion of pulse rise TOF which increases the resolution (Fig. 5) by emphasizing that portion of the pulse in which the cell predominates in changing the signal. Linearity and pulse height independence have been demonstrated.

The circuit is designed to stand independently, so it can be used as an outboard addition to any laser flow cytophotometer. It has, to date, been tested on the Ortho Instruments Cytofluorograph and the Becton-Dickinson FACS-II. It works equally well with broad and narrow laser beams and is therefore particularly suitable for use in quantitative cell light scattering experiments, which require the entire cell to be illuminated instantaneously. The pulse rise TOF feature is especially useful in such cases.

In biological experiments the system has been shown to demonstrate nuclear size changes through the human cell life cycle and consequent to virus infection. Absolute and relative N/C ratios have been measured. Continued application of this cell and nuclear sizing method in virology, immunology, oncology, cytopathology, and cell biology is anticipated.

ACKNOWLEDGMENTS

The authors thank Mr. Robert Habbersett who designed and built the electronic circuitry. We also thank Mr. Richard Hiebert for his extensive testing of this circuitry.

LITERATURE CITED

1. Dulbecco R, Freeman G: Plaque production by the polyoma virus. Virology 8:396, 1954

2. Gledhill BL, Lake S, Steinmetz LL, Gray JW, Crawford JR, Dean PN, Van Dilla MA: Flow microfluorometric analysis of sperm DNA content: effect of cell shape on the fluorescence distribution. J Cell Physiol 87:367, 1976

3. Leary JF: Laser light scattering detection and characterization of virally infected and transformed mammalian cells. Ph.D. thesis, Pennsylvania State University, University Park, 1977

4. Leary JF, Notter MFD, Todd P: Laser flow cytophotometric immunoperoxidase detection of herpes simplex virus type 2 antigens in infected cultured human cells. J Histochem Cytochem 24:1249, 1976

5. Leary JF, Todd P: Laser Cytophotometric detection of variations in spatial distribution of concanavalin-A cell surface receptors following viral infection. J Histochem Cytochem 25:908, 1977

6. Sharpless T, Traganos F, Darzynkiewicz Z, Melamed MR: Flow cytofluorometry: discrimination between single cells and cell aggregates by direct size measurements. Acta Cytol 19:577, 1975

7. Sharpless T, Melamed MR: Estimation of cell size from pulse shape in flow cytofluorometry. J Histochem Cytochem 24:257, 1976

8. Steinkamp JA, Crissman HA: Automated analysis of DNA, protein and nuclear to cytoplasmic relationships in tumor and gynecologic specimens. J Histochem Cytochem 22:616, 1974

9. Steinkamp JA, Hansen KM, Crissman HA: Flow microfluorometric and light scatter measurement of nuclear and cytoplasmic size in mammalian cells. J Histochem Cytochem 24:292, 1976

10. Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG: The unlabeled antibody enzyme method of immunochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-anti-peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315, 1970

11. Wheeless LL, Patten SF: Slit-scan cytofluorometry. Acta Cytol 17:333, 1973

12. Wheeless LL, Hardy JA, Balasubramanian J: Slit-scan flow system for automated cytopathology. Acta Cytol 19:45, 1975

FIGURE CAPTIONS

Figure 1. Time-of-flight cell sizing method. Cell diameter as measured by time-of-flight sizing depends on the choice of thresholds which determine what fraction f of the actual cell diameter d is measured and on the laser beam width W. In fixed threshold time-of-flight systems f is an unknown and varying fraction of the cell diameter. In this constant fraction time-of-flight sizing method f is a known constant fraction of the cell diameter. Since resolution of pulse width time-of-flight measurements decreases with large laser beam widths W, pulse rise-time measurement can be used to make the effective beam width W smaller thereby increasing the sizing resolution capability of the system.

Figure 2. Circuit for the measurement of the width of a voltage pulse between two fractions of its peak amplitude (simplified diagram). The input signal is split into two identical pulses so that a copy of the original pulse can be compared at a later time with constant fractions of itself. The peak sense and hold circuit "C" registers the peak pulse amplitude and holds this value for the duration of the measurement process. This peak value is reduced by potentiometers to desired threshold levels which can serve as reference inputs to the voltage comparators "F" and "G." Several microseconds after the peak sense and hold circuit records the peak amplitude, the input pulse passes through the delay line and is restored to its original amplitude by the amplifier. It becomes the second input "E" to the voltage comparators. The state of the voltage comparator "G" changes to the "on" state when the delayed replica of the original pulse has a voltage

between the chosen thresholds, and the state of the comparator returns to its original "off" value when the voltage pulse amplitude is outside of the chosen thresholds. Thus a voltage pulse is produced which has a width (time interval) equal to the width of the original pulse between the desired thresholds. A capacitor is then charged during this time interval to produce a voltage amplitude proportional to time (time to amplitude conversion, TAC).

Figure 3. Nuclear diameter (fluorescence time of flight) vs. pulse amplitude plot for Chinese hamster G2 cells counted with the cytofluorograf while maintaining the input pulses to the TOF circuit above 3.5 volts and varying the signal intensity by varying laser power and acriflavine staining procedure.

Figure 4. Calibration of light scatter pulse width time-of-flight sizing of microspheres. Pulse widths were measured from 5 percent peak amplitude threshold on the rising side of the signal to 5 percent of the peak amplitude threshold on the trailing side of the signal. These measurements give low resolution but wide dynamic range and were made on the Ortho Instruments Cytofluorograf (Model 4802A). Similar resolution has been achieved on the Becton-Dickinson FACS II.

Figure 5. Calibration of high resolution light scatter pulse rise time-of-flight sizing of microspheres between 10 and 35 percent of the peak amplitude thresholds on the rising side of the signal. These measurements were made on the Becton-Dickinson FACS II. Similar resolution has been achieved on the Ortho Instruments Cytofluorograf (Model 4802A).

Figure 6. DNA content distributions ("PHA", solid lines) of acriflavine stained cultured human embryonic lung (HEL) fibroblasts and nuclear diameter distributions ("TOF", dashed lines) determined on the same cell suspensions. 6 a (upper panel): The nuclear diameter distribution reflects the distribution of cells through the life cycle. 6b (lower panel): The nuclear diameter distribution reflects distortions in nuclear size caused by 18 hr of infection with herpes simplex virus type 2 (HSV-2).

Figure 7. Diagrammatic representation of a method for measuring N/C ratios of cells with cytoplasm stained either immunoenzymatically or by another histochemical staining method with a light-absorbing stain and nucleus stained with a DNA-specific fluorescent stain. Distinction between cells of the same size and N/C ratio depends on simultaneous pulse-height independent scatter and fluorescence TOF measurements (which are unaffected by the presence of the light absorbing stain or by variations in fluorescent stain intensity) and large-angle light scatter intensity which is decreased in cells stained with the light absorbing stain.

Figure 8. Simultaneous fluorescent and scatter time-of-flight measurements of nuclear size and cell size to obtain N/C ratios according to the scheme diagrammatically presented in figure 7. The life cycle of cultured human carcinoma (HEp-2) cells infected with herpes simplex virus type 2 (HSV-2) is perturbed as shown by the difference in N/C ratios as compared to the uninfected control cells. In this two-parameter distribution nuclear diameter is plotted horizontally and cell diameter vertically, increasing downward. Each 2-parameter microprocessor channel contains a number which, when multiplied

by 32 gives the number of cells in that channel. Regions of channels with 224 or more cells/channel are outlined to show peaks. There are 2 such regions, A and B in the infected cell distribution and 3 regions, C, D, and E, in the uninfected cell distribution. A simple ratio of nuclear and cell TOF signals will not yield a true N/C ratio. Therefore, the peak channels in each distribution must be converted to diameters by the use of calibration plots such as those in Figs. 4 and 5. Note that the HSV-2 infected cells are larger and that population C consists of bare nuclei, as indicated by N/C of nearly 1.0. This experiment used the pulse rise TOF method with thresholds set at 5% and 35% of maximum peak height.

FIGURE 1

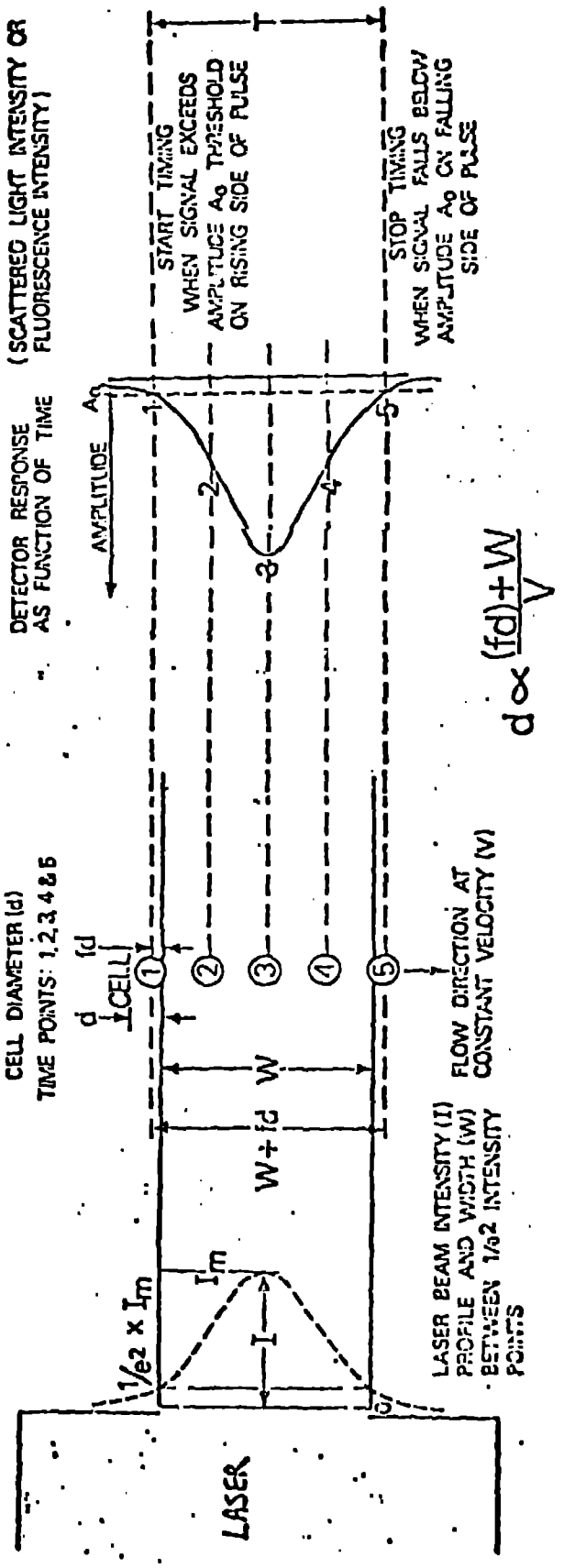
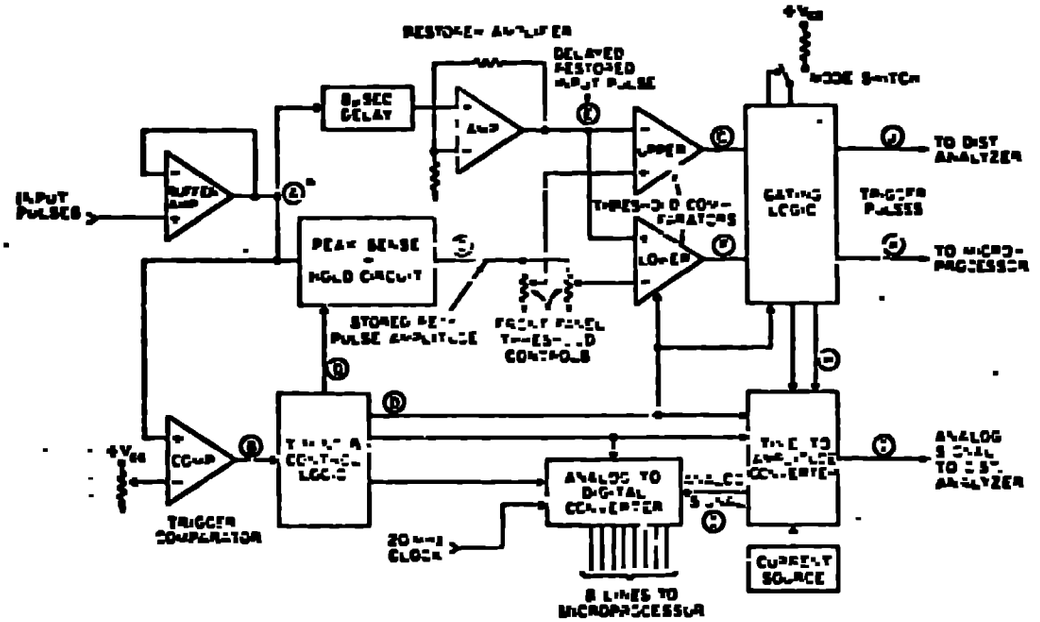

DETECTOR RESPONSE AS FUNCTION OF TIME
(SCATTERED LIGHT INTENSITY OR FLUORESCENCE INTENSITY)
AMPLITUDE
A_0
START TIMING WHEN SIGNAL EXCEEDS AMPLITUDE A_0 THRESHOLD ON RISING SIDE OF PULSE
STOP TIMING WHEN SIGNAL FALLS BELOW AMPLITUDE A_0 ON FALLING SIDE OF PULSE
1
2
3
4
5
$\propto \frac{(fd)+W}{V}$
CELL DIAMETER (d)
TIME POINTS: 1,2,3,4&5
d
fd
CELL
W
W+fd
I_m
$1/e^2 \times I_m$
I
LASER
LASER BEAM INTENSITY (I) PROFILE AND WIDTH (W) BETWEEN 1/e2 INTENSITY POINTS
FLOW DIRECTION AT CONSTANT VELOCITY (V)
d

FIGURE 2


RESTORER AMPLIFIER
DELAYED RESTORED INPUT PULSE
8 μSEC DELAY
MODE SWITCH
INPUT PULSES
BUFFER AMP
PEAK SENSE & HOLD CIRCUIT
THRESHOLD COMPARATORS
UPPER
LOWER
GATING LOGIC
TRIGGER PULSES
TO DIST ANALYZER
TO MICRO-PROCESSOR
STORED PEAK PULSE AMPLITUDE
FRONT PANEL THRESHOLD CONTROLS
TIME TO AMPLITUDE CONVERTER
ANALOG TO DIGITAL CONVERTER
ANALOG SIGNAL TO DIST. ANALYZER
TRIGGER COMPARATOR
20 MHz CLOCK
CURRENT SOURCE
8 LINES TO MICROPROCESSOR

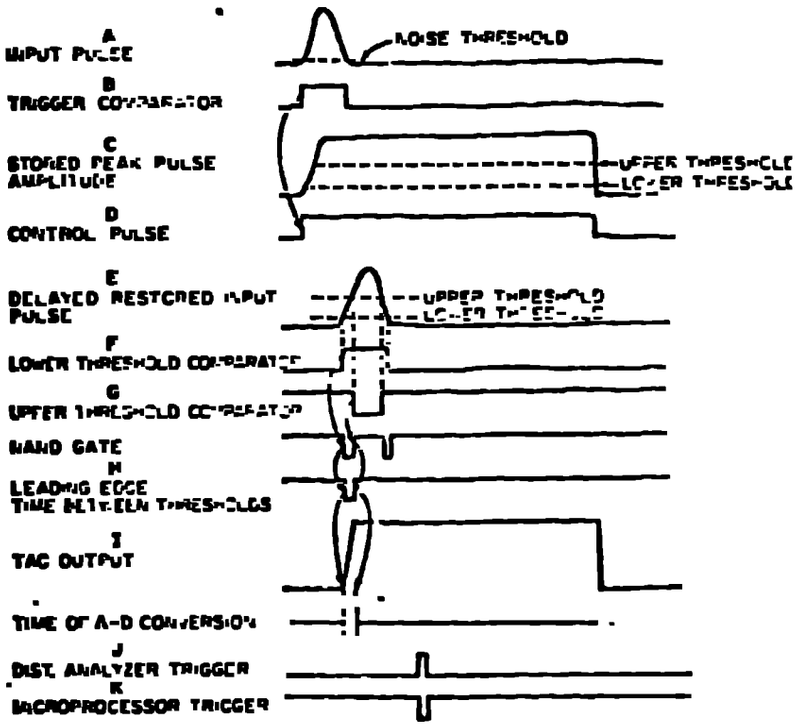
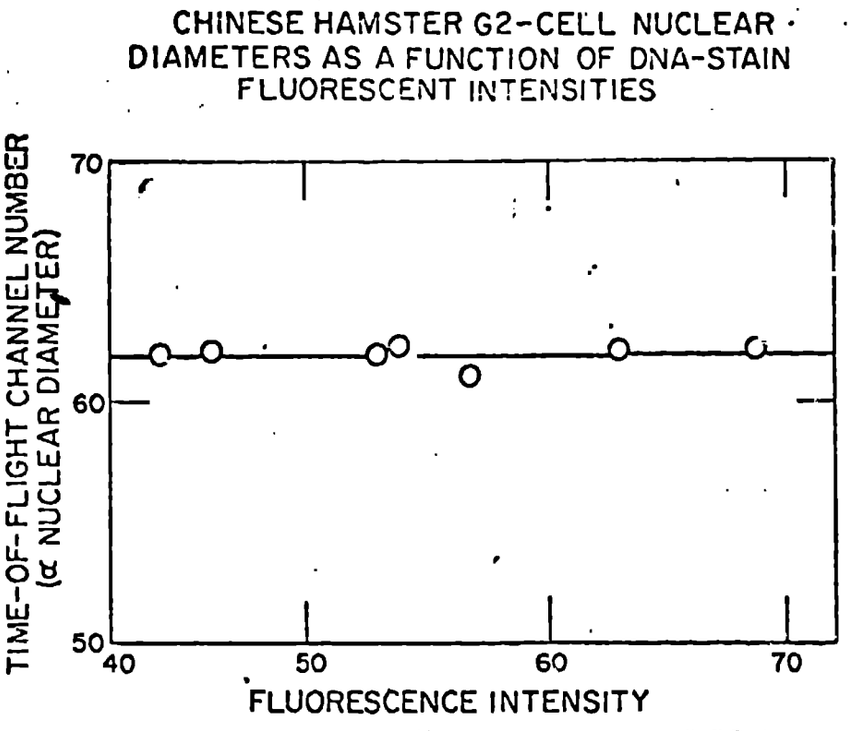
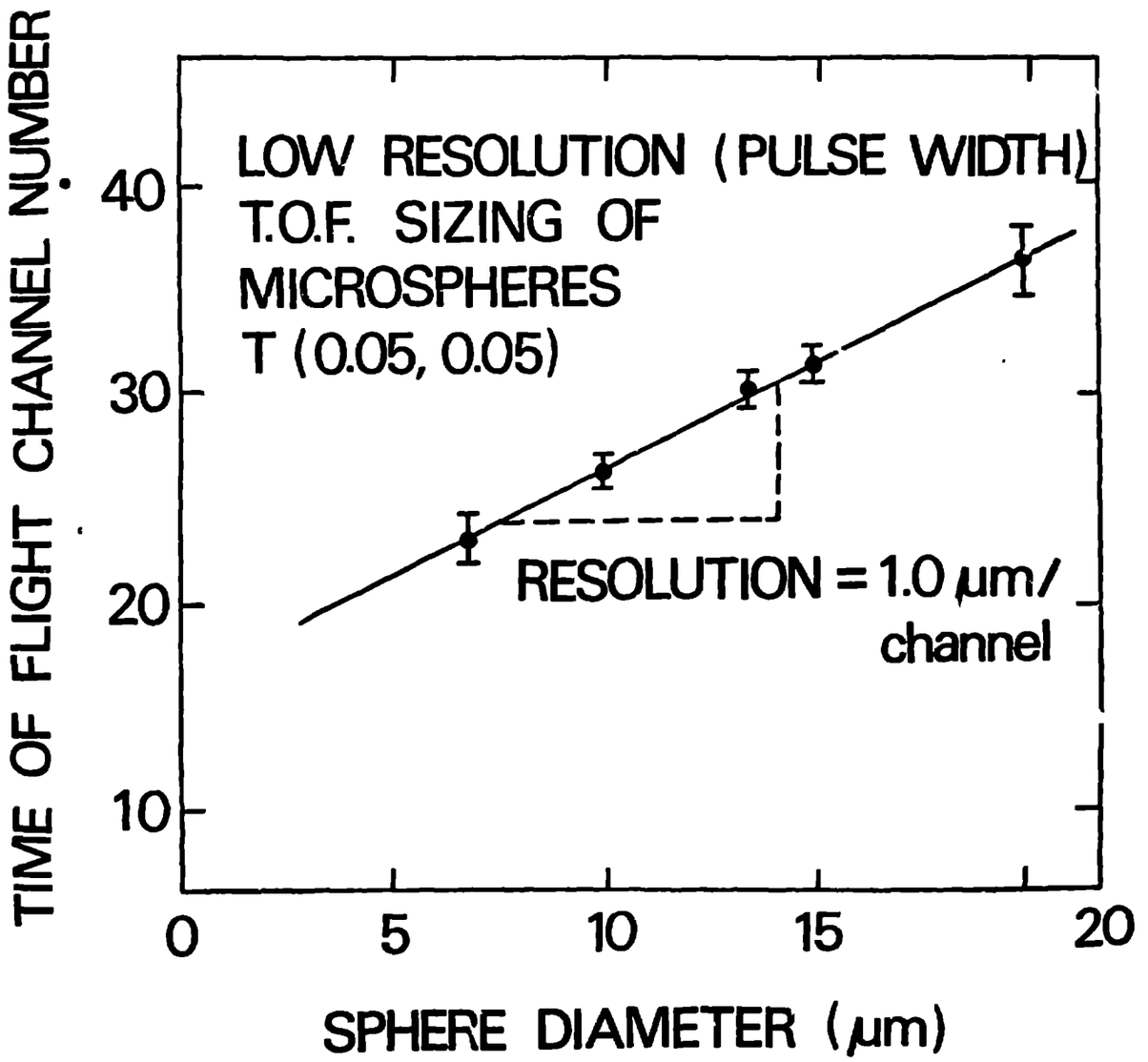

A
INPUT PULSE
NOISE THRESHOLD
B
TRIGGER COMPARATOR
C
STORED PEAK PULSE AMPLITUDE
UPPER THRESHOLD
LOWER THRESHOLD
D
CONTROL PULSE
E
DELAYED RESTORED INPUT PULSE
F
LOWER THRESHOLD COMPARATOR
G
UPPER THRESHOLD COMPARATOR
H
AND GATE
LEADING EDGE TIME BETWEEN THRESHOLDS
I
TAC OUTPUT
TIME OF A-D CONVERSION
J
DIST. ANALYZER TRIGGER
K
MICROPROCESSOR TRIGGER

FIGURE 3


CHINESE HAMSTER G2-CELL NUCLEAR DIAMETERS AS A FUNCTION OF DNA-STAIN FLUORESCENT INTENSITIES
TIME-OF-FLIGHT CHANNEL NUMBER (α NUCLEAR DIAMETER)
70
60
50
40
50
60
70
FLUORESCENCE INTENSITY


LOW RESOLUTION (PULSE WIDTH)
T.O.F. SIZING OF
MICROSPHERES
T (0.05, 0.05)
RESOLUTION = 1.0 μm/
channel
TIME OF FLIGHT CHANNEL NUMBER
40
30
20
10
0
5
10
15
20
SPHERE DIAMETER (μm)

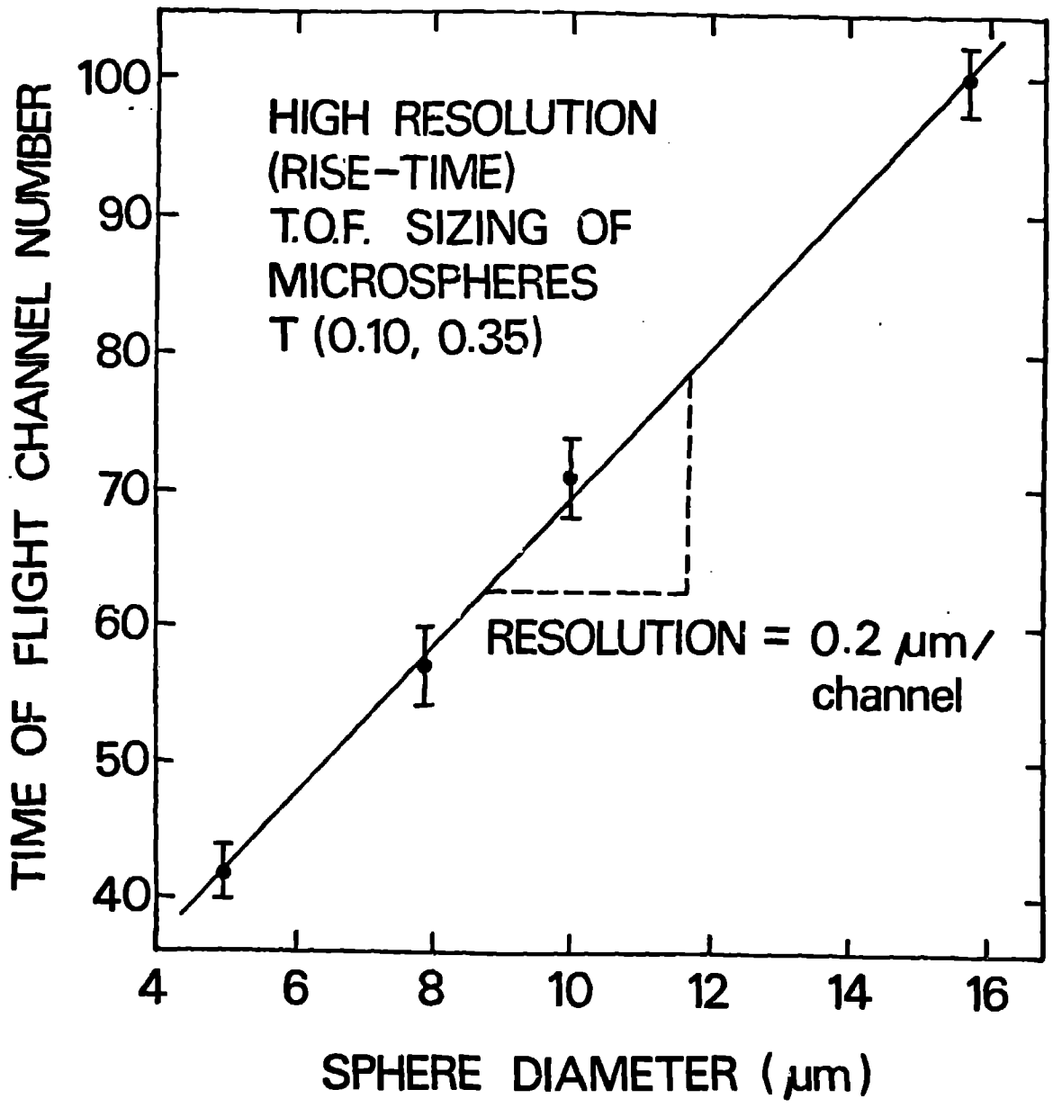

HIGH RESOLUTION
(RISE-TIME)
T.O.F. SIZING OF
MICROSPHERES
T (0.10, 0.35)
RESOLUTION = 0.2 μm/
channel
TIME OF FLIGHT CHANNEL NUMBER
100
90
80
70
60
50
40
4
6
8
10
12
14
16
SPHERE DIAMETER (μm)

FIGURE 6

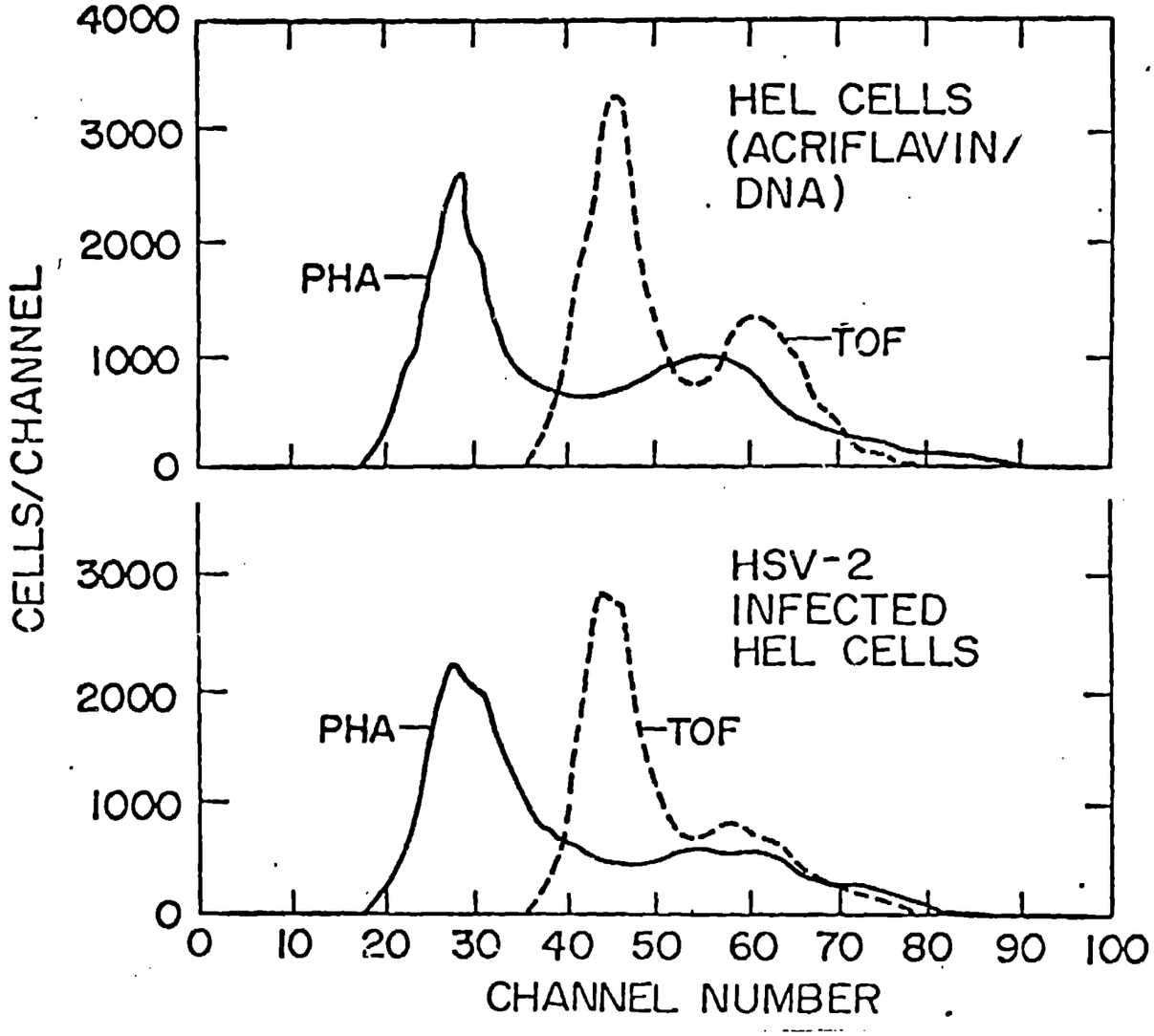
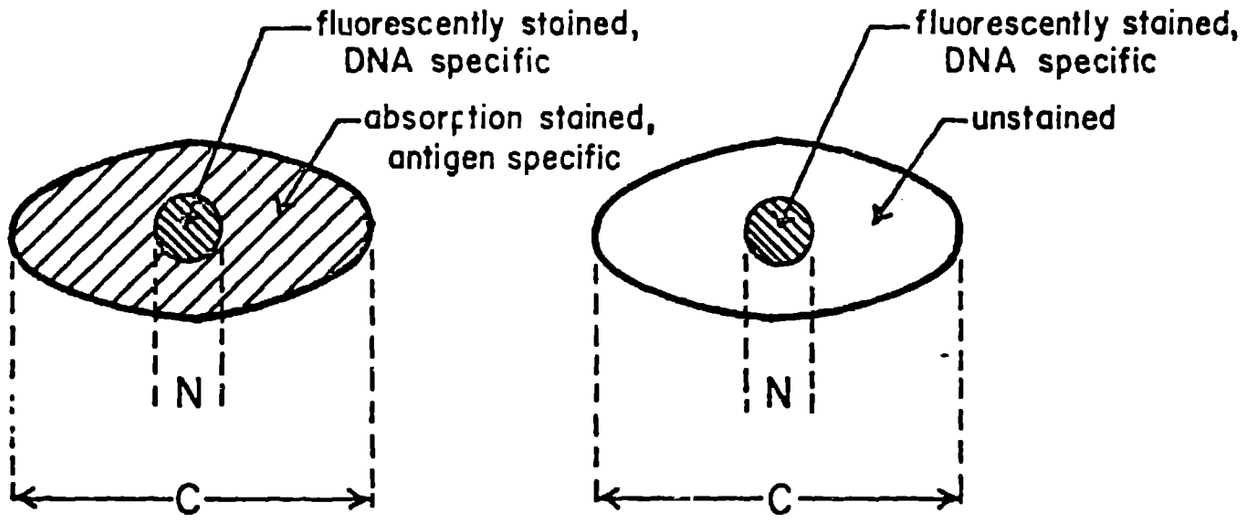

4000
3000
2000
1000
0
HEL CELLS
(ACRIFLAVIN/
DNA)
PHA
TOF
CELLS/CHANNEL
3000
2000
1000
0
HSV-2
INFECTED
HEL CELLS
PHA
TOF
0 10 20 30 40 50 60 70 80 90 100
CHANNEL NUMBER

FIGURE 7

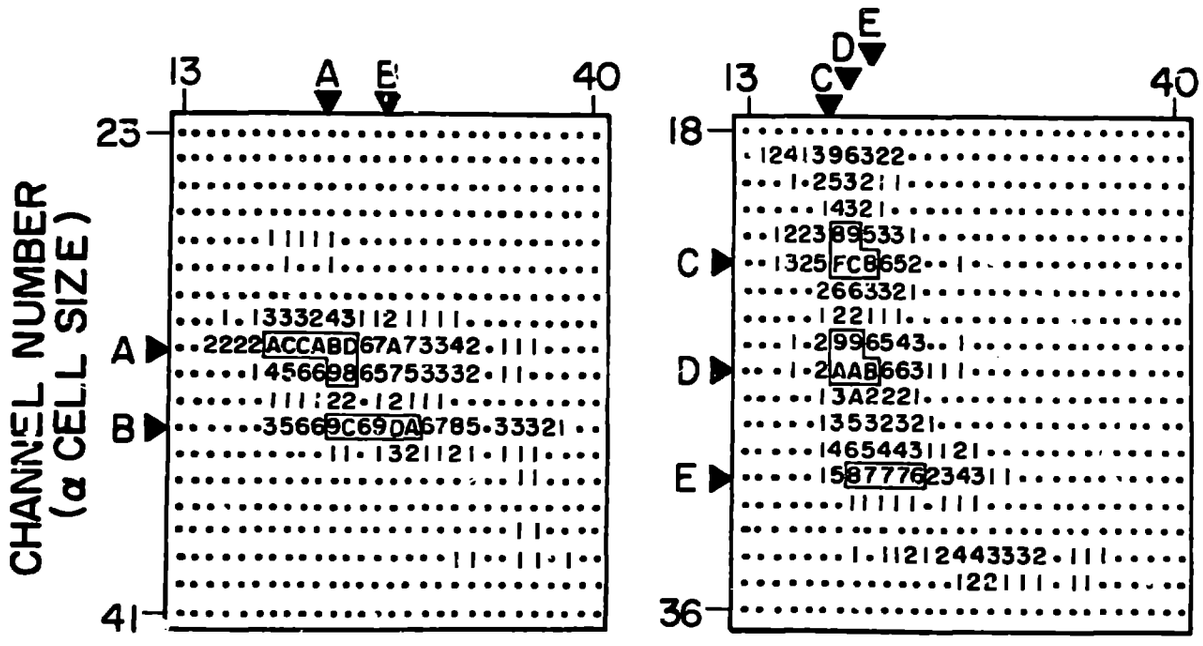
NUCLEAR SIZE / CELL SIZE AS MEASURED BY
PULSE HEIGHT INDEPENDENT TIME-OF-FLIGHT



N measured by fluorescence time-of-flight

C measured by scatter time-of-flight

NUCLEAR/CYTOPLASMIC RATIOS OF INFECTED AND UNINFECTED CELLS T(0.05, 0.35)



HSV-2 INFECTED HEp-2 CELLS

$[N/C]_A = 0.705$

$[N/C] = 0.720$

UNINFECTED HEp-2 CELLS

$[N/C]_C = 0.977$

$[N/C]_D = 0.764$

$[N/C]_E = 0.687$